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Quantitation of polar analytes using column-switching: Application to oxycodone and three metabolites in human plasma

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ABSTRACT

We present herein a sensitive and selective assay for the determination of oxycodone and its main metabolites, oxymorphone, noroxycodone and noroxymorphone in human plasma, using column-switching and liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). Sample preparation comprised protein precipitation with perchloric acid. After neutralization, the supernatant was injected without any evaporation step onto a polymeric, pH-resistant cartridge (HySphere Resin GP 10–12 μm) for sample clean-up (Prospekt II). The latter operation was achieved by using alkaline conditions to ensure retention of analytes and methanol for matrix interference removal. More than two hundred plasma samples could be analyzed with a single cartridge. Analytes were desorbed in the backflush mode and were separated on a conventional reversed phase column (XTerra MS 4.6 × 50 mm, 3.5 μm), using an acidic mobile phase (i.e. containing 0.1% of formic acid). Mass spectrometric detection was achieved with a 4000 Q TRAP equipped with an atmospheric pressure chemical ionization (APCI) source, in positive ionization mode, operated in the selected reaction monitoring mode (SRM). Starting from a plasma volume of 250 μl, quantification ranges were 25–10,000 pg/ml for OXM and NOXM and 50–10,000 pg/ml for OXC and NOXC. Accuracy was found to be within 98% and 108% and precision better than 7%. Replicate determination of incurred or study samples ensured the method to be reproducible and usable for clinical studies.

1. Introduction

The popularity of liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) for bioanalysis studies is largely due to the selectivity and sensitivity allowed by triple quadrupole instruments (QqQ) operated in the selected reaction monitoring mode (SRM) [1]. For assays requiring reasonable sensitivities (i.e. in the ng/ml range), the performances of current commercial instruments allow the method development to be relatively limited: generic protein precipitation (PPT) procedures and fast LC gradients are usually sufficient for most cases. Nevertheless, challenges arise when sensitivities at the low pg/ml have to be reached. Chromatographic separation and sample preparation have then to be considered carefully, especially to cope with matrix effects and interferences issues. As various samples preparation strategies (solid phase extraction, liquid–liquid extraction, etc.) have found acceptance, chromatography is mostly performed in the reversed phase mode (RP-LC), due to its reproducibility. RP-LC is well suited for non-polar compounds, since the retention is governed by hydrophobic interactions. The retention of an analyte can be correlated with its log *D*, which is a measure of lipophilicity (hydrophobicity) relative to pH: the greater the value, the greater the hydrophobicity [2]. Limitations of RP-LC arise with polar compounds, i.e. having log *D* values close to zero or negative, and exhibiting thus little or no retention. Their elution thus occurs in the void volume, which accentuates possible matrix effects and ion suppression. Hence, their analysis is non-trivial, and this represents an important issue in bioanalysis. Indeed, besides polar drugs, metabolites could also possibly fall within this scope, since, in order to facilitate elimination, detoxification reactions occurring in living organisms aim at forming more polar derivatives.

Several strategies with mass spectrometric detection have been proposed to improve retention polar analytes such as (i) ion pairing (ii) RP and (iii) non-RP separations. First, in the case of charged analytes the addition of appropriate volatile counter-ions in the mobile phase leads to the formation of a neutral, hydrophobic ion pair. Examples include perfluorinated carboxylic acids (heptafluorobutanoic, nonafluoropentanoic, tridecafluoroheptanoic and pentadecafluorooctanoic acid), which were used by Gao et al. for the determination of methadone in human plasma

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[3], and hexylamine, which was used by Coulier et al. to analyze endogenous metabolites in microorganism extracts [4].

A second angle would be considering alternative RP materials designed to offer different selectivities and to improve the retention. Among those, porous graphitized columns rely on hexagonal arrangements of carbon atoms into sheets. In addition to dispersive (i.e. hydrophobic) interactions, graphite is responsible of for charge-induced interactions between polarizable or polar groups of the analyte, which leads to an improved retention for polar and ionic compounds [5]. Bioanalytical methods for polar compounds were reported for nucleobase derivatives [6–9], gluthatione derivates [10] and other polar drugs [11–14].

A third approach would be using different retention mechanisms, i.e. non-RP separations. Ion chromatography involves electrostatic interactions for the separation of (polar) charged compounds [15]. Typical stationary phases are grafted with strong acid/base functions, and imply the use of high salt concentration, non-MS-compatible mobile phases for elution. Coupling with MS is nevertheless possible by the use of a post-column suppressor, for background ion removal. This setup has mostly given rise to applications dealing with inorganic ions, often in environment (water) samples. Its potential for small organic molecules in plasma (chlorophacinone) [16], serum (valone) [17] and (clam) tissues (chlorophenols) [18] has been demonstrated, but remains marginal. On another hand, weak cation exchange, unlike strong cation exchange, allows the use of low ionic strength, MS-compatible mobile phases. Those columns have been used by Wang et al. for the determination of catecholamines [19].

Hydrophilic interaction chromatography (HILIC), on the other hand, relies on stationary phase materials (usually silica-based) which, when used with an aqueous/organic eluent, gives rise to the formation of a stagnant, water-enriched layer on its surface [20]. The retention of uncharged compounds is currently believed to involve the partition between the immobilized hydrophilic layer and the relatively hydrophobic mobile phase. In addition, possible adsorption, hydrogen bonding or electrostatic interactions with charged sites (for instance deprotonated silanols) also seem to contribute to the retention mechanism [21]. Despite the fact that HILIC is not well understood from a mechanistic point of view, it has become popular for the separation of polar mixtures and given rise to much larger applications than the previous approaches. Orthogonal with RP-LC, HILIC also allows the use of the same mobile phases as RP-LC, which makes the transition from the classical RP-LC to HILIC very convenient. For those reasons, HILIC is often referred as a "reversed RP". Numerous applications have been reported for quantitative bioanalysis, as recently reviewed by Hsieh [22].

Oxycodone (OXC) is an opioid receptor agonist largely prescribed for pain management [23]. It undergoes an extensive hepatic metabolism, which leads to three major circulating metabolites (Fig. 1). Noroxycodone (NOXC) is formed by CYP3A4- and CYP3A5-mediated N-demethylation. Another isoform of cytochrome P450, CYP2D6, catalyzes O-demethylation and generates oxymorphone (OXM). Lastly, noroxymorphone (NOXM) is a secondary metabolite of OXC essentially formed by O-demethylation (CYP2D6-mediated) of NOXC [24].

Because of its cytochrome-mediated metabolism, significant variations of plasma concentrations of OXC and of its metabolites can be observed between patients. First, important inter-individual variations of levels and activities of cytochromes have been reported, which will impact circulating levels of the drug [25]. Second, because cytochromes are susceptible to be induced (CYP3A) or inhibited (CYP3A and 2D6), drug interactions can modify the drug effect [26]. Third, CYP2D6 exhibits a genetic polymorphism, which categorizes the population in ultra-rapid, extensive, intermediate, and poor metabolizers [27]. The latter group, which represents up to 10% of Caucasians, exhibits only weak or lack any enzymatic activity. Various LC-based methods have been proposed for the determination of OXC, and some of its metabolites [28–30], but among those, only a recent one [31] allowed the simultaneous determination of the four analytes, which is challenging due to the polarity of OXM and NOXM. The total chromatographic run time was nevertheless 25 min and sample preparation (solid phase extraction) performed off-line. Moreover, for clinical purposes, the limit of quantification has to be in the sub ng/ml range. Column-switching, which was introduced almost 30 years ago by Roth et al. [32], is an elegant and automated approach to achieve high sensitivity. It takes advantage of a first trapping column for sample enrichment and clean-up, and of a second analytical column for separation and further quantitation. Moreover, Bourgogne et al. reported the use of an on-line solid phase extraction (SPE) system with multiple uses of a single SPE cartridge on a Prospekt system from Spark Holland, operating thus in a column-switching fashion [33]. To investigate the impact of CYP2D6 and CYP3A activities on the pharmacokinetics of immediate release oxycodone a column-switching LC–MS/MS (Prospekt II) assay for the determinations of OXC, OXM, NOXC and NOXM in human plasma at the low pg/ml level was developed. Neuvonen et al. investigated the stability of the analytes in human plasma (freeze–thaw cycles, 5 h storage at bench temperature and two months storage at −20°) and did not report any degradation under the conditions studied [31].

2. Experimental

2.1. Chemicals and reagents

NOXM, OXM, NOXC, OXC, OXM-d_3 and OXC-d_3 were obtained from Cambridge Isotope Laboratories (Innerberg, Switzerland) and were supplied as 1 mg/ml (OXM, NOXC, OXC) and 0.1 mg/ml (NOXM, OXM-d_3 and OXC-d_3) solutions in methanol. Methanol and acetonitrile were obtained respectively from Sigma–Aldrich (Buchs, Switzerland) and Biosolve (Valkenswaard, The Netherlands). Formic acid and perchloric acid (70%) were obtained respectively from Merck (Darmstadt, Germany) and Fluka (Buchs, Switzerland). Ammonium hydroxide solution (25%) and ammonium acetate were obtained respectively from Fluka (Buchs, Switzerland) and Acros organics (Geel, Belgium). Citrate plasma and study samples were provided by the Geneva University Hospital (Geneva, Switzerland).

2.2. Preparation of spiked human plasma samples

Doping stock solutions of the analytes of interest were first prepared for spiking purpose. They consisted of a mixture of NOXM, OXM, NOXC and OXC in a mixture of H_2O/MeOH 1/1. Calibrator standard and quality control (QC) solutions were obtained by mixing 20 μl of appropriate doping solutions to 980 μl of human plasma, yielding the following final concentrations: 25, 50, 100, 250, 500, 1000, 5000 and 10,000 pg/ml.

2.3. Protein precipitation

Plasma proteins were precipitated by adding 250 μl of a mixture of OXM-d_3 (500 pg/ml) and OXC-d_3 (500 pg/ml) in $(HClO_4)_{aq}$ 5% to 250 μl of spiked plasma. After centrifugation (10 min – 16,435 × *g*), the supernatant was removed and neutralized with 175 μl of NH_4OH 10%.

2.4. Column-switching

Column-switching was performed using a Prospekt II (Spark Holland, The Netherlands), with HySphere Resin GP 10–12 μm (Spark Holland, The Netherlands) cartridges for analyte trapping

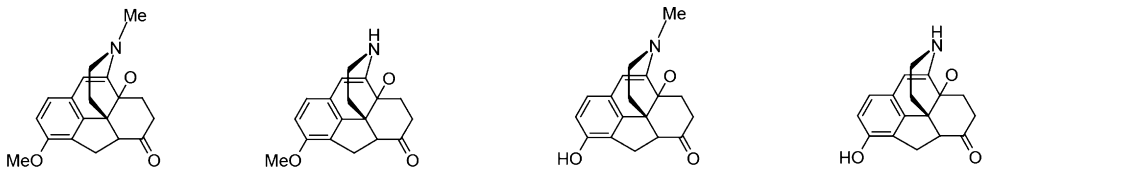

Fig. 1. Chemical structures and log D_{calc} of analytes.

Table 1
Prospekt II procedure.

Step	Solvent	Volume (ml)	Flow rate (ml/min)
Cartridge conditioning	MeOH/H_2O 9/1	2	4
Equilibration	NH_4OAc buffer 50 mM pH 9.25 in H_2O	2	4
Sample injection (500 μl)	–	–	–
Clean-up	NH_4OAc buffer 50 mM pH 9.25 in H_2O/MeOH 85/15	2	2
Pre-conditioning	H_2O	1.5	2
Elution onto column (backflush, 50 s)	–	–	–
Cartridge wash	MeOH/H_2O 9/1	3	4

and sample clean-up (Fig. 2). The procedure, detailed in Table 1, involves an injection volume of 500 μl. Clean-up solutions were prepared by diluting tenfold ammonium buffer 500 mM pH 9.25 with methanol and water so that the final mixture is ammonium buffer 50 mM in H_2O/MeOH (v/v).

2.5. Liquid chromatography

The HPLC system used for RP-LC comprised two LC-10 ADvp pumps (Shimadzu, Reinach, Switzerland) operated in high-pressure gradient mode and a XTerra MS C18 4.6 × 50 mm, 3.5 μm (Waters, Milford, MA, USA) with a SecurityGuard Cartridge, C18 4 × 2 mm (Phenomenex, Torrance, CA, USA). Mobile phases were A: H_2O + 0.1% HCOOH and B: MeCN + 0.1% HCOOH. Separation was achieved at 1 ml/min, in gradient mode (Table 2).

HILIC chromatography was performed using a Rheos Allegro pump (Thermo-Fischer, Reinach, Switzerland) and an Acquity UPLC BEH HILIC 2.1 × 100 mm, 1.7 μm column (Waters, Milford, MA, USA). The separation was achieved in isocratic mode, using a mobile phase comprising ammonium formate buffer (10 mM, pH 4.25) in H_2O/MeCN 8/92 (v/v), at a flow rate of 1 ml/min.

2.6. Mass spectrometry

Detection of the analytes was performed with a 4000 Q TRAP (Applied Biosystems, MDS Sciex, Concord, ON, Canada), equipped with an atmospheric pressure chemical ionization source (APCI), operated in positive ionization mode. Conditions were: curtain gas = 20 psi, gas 1 = 50 psi, nebulizer current = 3 μA, source temperature = 600 °C, declustering potential = 85 V. The transitions and the corresponding collision energies (CE) used for SRM were the following – NOXM: m/z 288.2 → m/z 213.3 (CE = 40 eV); OXM: m/z 302.2 → m/z 227.3 (CE = 38 eV); NOXC: m/z 302.3 → m/z 187.3 (CE = 35 eV); OXC: m/z 316.3 → m/z 241.2 (CE = 40 eV); OXM-d_3: m/z 305.2 → m/z 230.0 (CE = 42 eV); OXC-d_3: m/z 319.3 → m/z 244.1 (CE = 41 eV). Nitrogen was used as collision gas at a setting (CAD) of 6.

3. Results and discussion

3.1. Mass spectrometric considerations

Due to better signal to noise ratios and sensitivities, atmospheric pressure chemical ionization (APCI) was chosen in preference to electrospray (ESI) as ionization source. MS/MS transitions of analytes were optimized based on collision energy and declustering potential parameters. For all four analytes the most abundant product ion observed is the loss of water (−18 u) (Fig. 3). This fragment was not selected for the SRM transitions because of the lack of selectivity of the water loss. As depicted in Fig. 3, OXM and NOXC show common fragment ions, but, among the most intense ones, only NOXC gives rise to the m/z 187 ion. Nevertheless, all the most intense fragment ions of NOXC are also common to OXM, which implies that, in absence of baseline separation, NOXC will bias the determination of OXM.

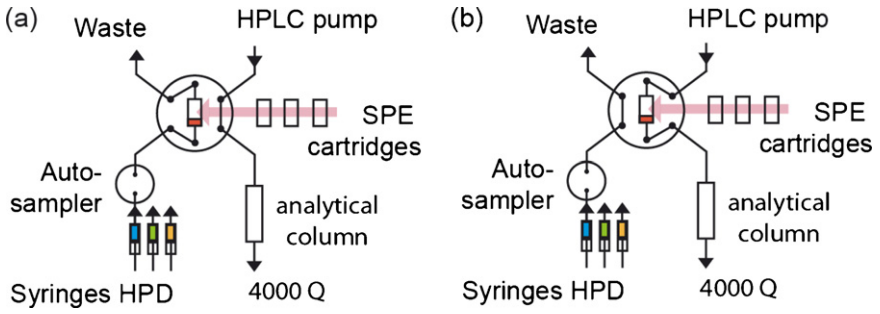

Fig. 2. Prospekt II configuration for trapping/clean-up step (left) and elution (right).

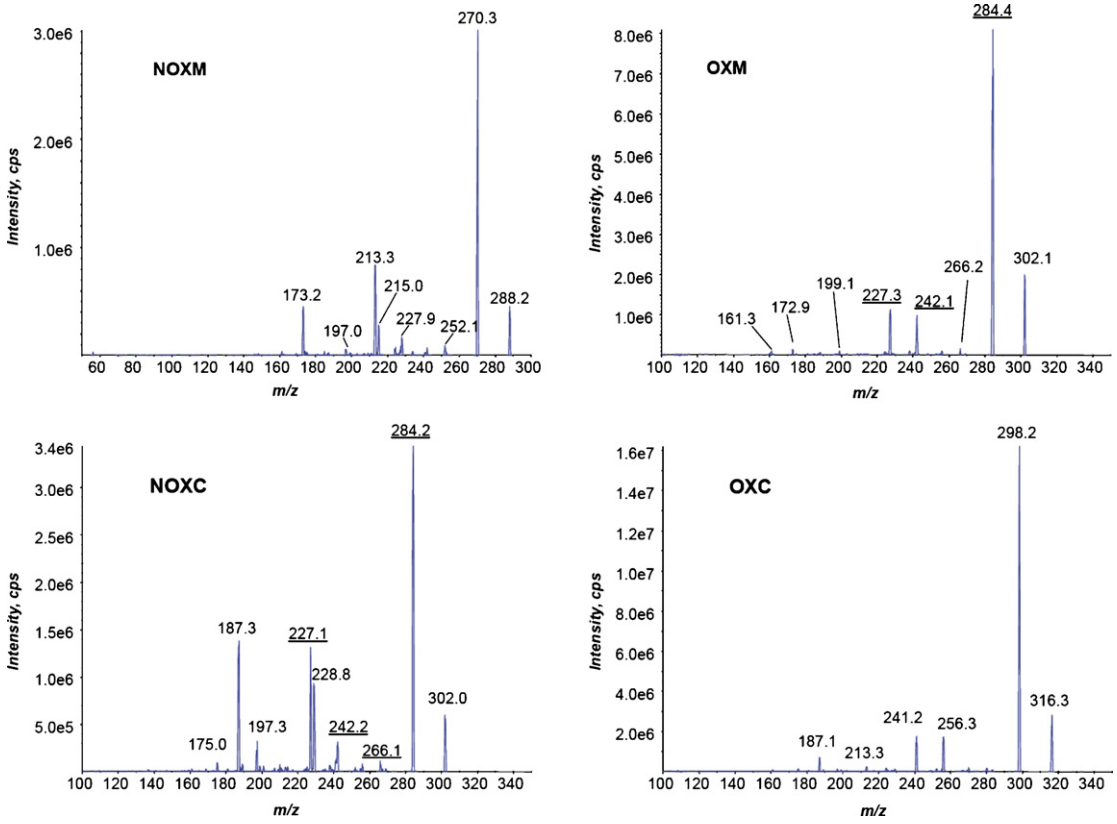

Fig. 3. MS/MS spectra of the analytes. Common fragments ions of OXM and NOXC are underlined.

3.2. Chromatographic separation

The first challenge is to ensure, due to their polar nature, sufficient retention of the analytes onto the analytical column. The situation is made more complicated by the fact that OXM and NOXC are isobaric species sharing common MS/MS fragments (Fig. 3), which makes the baseline chromatographic separation for OXM and NOXC mandatory. HILIC is nowadays one of the most popular approaches for compounds which show limited or no retention on RP materials. In the same way as RP-LC, elution is also achieved with water/organic solvent (typically acetonitrile) mixtures. The retention mechanism, usually referred to as reversed RP-LC, is nevertheless orthogonal with RP-LC, the elution order having been demonstrated to be roughly the opposite of RP-LC [20]. This makes the transition from RP-LC to HILIC and method development relatively convenient. Moreover, if volatile pH-modifiers are used, the interfacing with MS is straightforward. In contrast with RP-LC, elution of analytes typically occurs with high organic content mobile phases, which gave HILIC the reputation to increase atmospheric pressure ionization (API) efficiency. For those reasons, it appeared justified to investigate HILIC.

Besides partition between the stagnant hydrophilic and mobile hydrophobic layers, retention is also based on electrostatic interactions and hydrogen bonding. It seems thus obvious that those interactions will be impacted by the charge state of the stationary phase. Thus, pH would be expected to be a key parameter for separation selectivity. For this purpose, several mobile phases of same MeCN/H_2O proportions (92/8, v/v) were prepared, with volatile buffers of equal concentration but of different pH (ammonium acetate 10 mM at pH 4.25, 4.75 and 5.25; ammonium formate at pH 3.25, 3.75 and 4.25). The separation was achieved without prior column-switching, in order to assess the intrinsic retention and separation efficiency of HILIC. Optimization criteria were a baseline separation of OXM and NOXC, the peak shape and the analysis time.

As anticipated, pH was found to be crucial to modify the separation selectivity. Optimal conditions were found to be ammonium formate 10 mM pH 4.25 in MeCN/H2O 92/8 (v/v). In these conditions, all peaks were baseline separated in less than 2.5 min (Fig. 4).

RP-LC experiments were conducted with an identical flow rate and ion source used for HILIC investigation (i.e. 1 ml/min and APCI). The RP-LC experiments were performed with the trapping cartridge on-line, because it was observed that the use of a cartridge allows additional, useful retention. Elution was achieved with a water/acetonitrile gradient. The use of formic acid (0.1%) as pH-modifier will be discussed in the column-switching section. Using this setting, baseline separation of the isobaric compounds was achieved within a similar time, with coelution of NOXM with OXM and of NOXC with OXC (Fig. 4). The latter situation is, from a mass spectrometric point of view, more favorable. Indeed, because OXC-d_3 and OXM-d_3 were used as internal standards (IS), they will compensate for possible matrix effects. In addition, using the same source and same source settings, RP-LC showed better sensitivities and better signal to noise ratios than HILIC did. In conclusion, the RP-LC conditions were chosen for the separation.

Table 2
RP-LC gradient.

Time (min)	% B
0.00	5
2.50	25
2.51	100
3.00	100
3.01	5

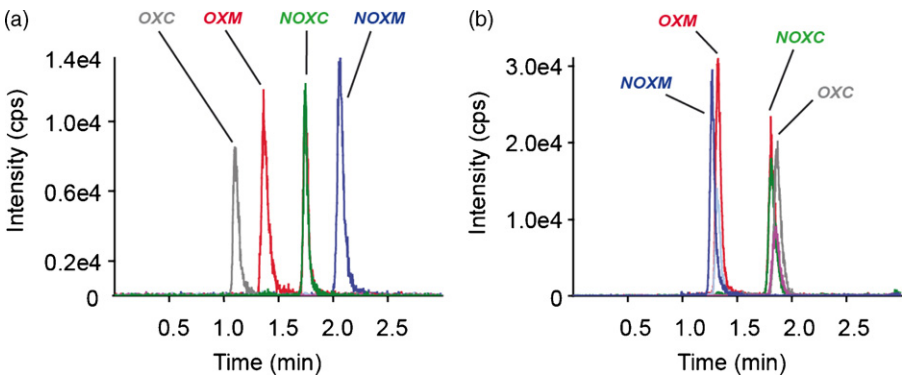

Fig. 4. Chromatograms obtained with HILIC (left) and RP-LC (right).

3.3. Column-switching optimization

3.3.1. Sample preparation

Prior to column-switching, protein precipitation (PPT) was performed in order to re-use the same cartridge several times in a column-switching fashion, as demonstrated by Bourgogne et al. [33]. Using organic solvents for PPT compromise analyte retention onto the trapping cartridge and require evaporation and reconstitution of the supernatant in an appropriate solvent, which is time consuming. In contrast, PPT achieved with a perchloric acid solution leads to an aqueous supernatant, which, after a simple alkalinization step (ammonia addition), can be readily injected onto the cartridge.

3.3.2. Analyte trapping

The use of alkaline injection conditions was indeed found to be crucial for an efficient analyte trapping. This is highlighted by $\log D_{calc}$ of analytes, which were predicted with a software program (Advanced Chemistry Development V8.14, ACD/Labs; accessed via SciFinder Scholar 2007) and are reported in Fig. 1. Values were found to be negative in acidic and neutral conditions: this suggests no retention on RP materials. In contrast, at pH 9, $\log D_{calc}$ were found to be positive for all compounds (except NOXM, close to zero), which suggested that the use of alkaline conditions would improve retention. Because usual RP materials are based on silica particles, the typical working range is limited to pH 2–8. Advantage was then taken of polymeric, pH-resistant cartridges, which allow the use of alkaline solutions. To ensure the proper retention of analytes, the cartridge was equilibrated with ammonium buffer, 50 mM, pH 9.25 before sample injection. Under these conditions, more than 90% of the analyte could be retained onto the trapping cartridges.

3.3.3. Matrix interference removal and analyte recovery

After trapping, and prior LC–MS/MS analysis, plasma samples undergo a clean-up step, which raises the questions of analyte recovery and endogenous interference removal. The sole use of an aqueous, alkaline solution (ammonium buffer 50 mM, pH 9.25) did not provide efficient clean-up (Fig. 5) and would jeopardize quantitation at the low pg/ml level, since endogenous interferences which may vary from sample to sample could interfere with the analytes. A large variability of endogenous inference profiles was indeed observed from one plasma sample to another (data not shown). The addition of various amounts of methanol (5%, 10%, 15%, 20% and 25%), with parallel assessment of corresponding recoveries, was thus investigated. Analyte recoveries were calculated as the mean

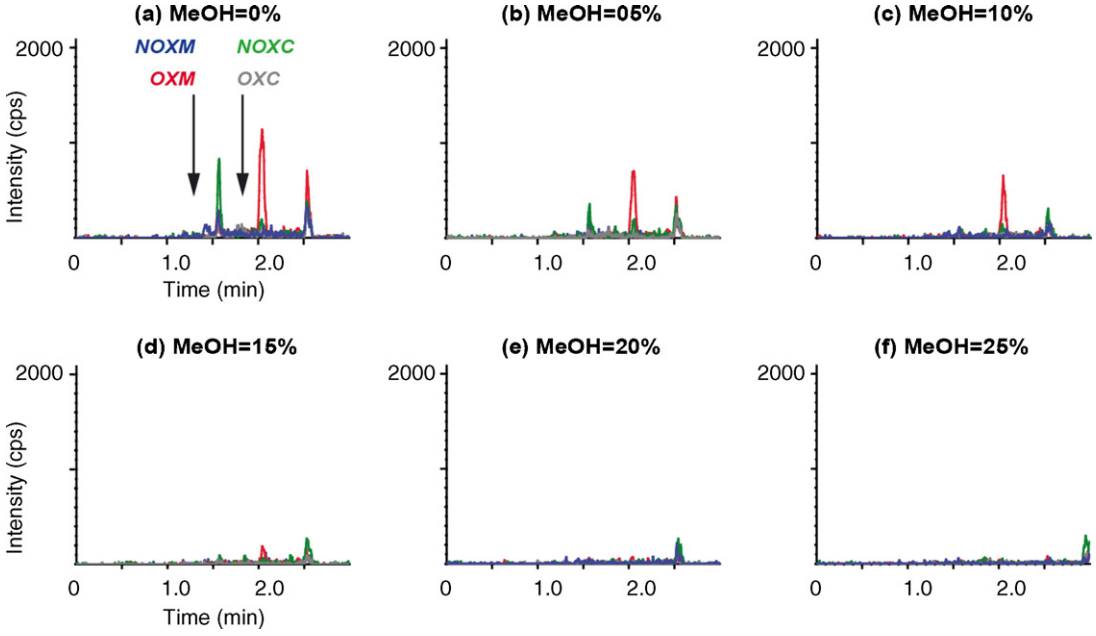

Fig. 5. Endogenous interferences removal: chromatograms of blank plasmas with different MeOH percentage of the clean-up solution. Arrows indicate retention times of the analytes.

Table 3
Precision and accuracy results.

Analyte	QC level	Expected concentration (pg/ml)	Calculated concentration (pg/ml)					Mean (n = 5)	Relative standard deviation	Accuracy
			Day 1			Day 2				
			Series 1	Series 2	Series 3	Series 4	Series 5			
NOXM	LLOQ	25	24.54	22.60	27.15	25.30	25.80	25.08	6.7%	100.3%
	Low	50	52.62	49.04	51.73	52.00	50.10	51.10	2.9%	102.2%
	Medium	500	497.0	518.6	494.7	489.3	484.5	496.8	2.6%	99.36%
	High	5000	4921	5068	4929	5265	5089	5054	2.8%	101.1%
OXM	LLOQ	25	26.93	27.65	24.85	25.82	23.50	25.75	6.4%	103.0%
	Low	50	48.56	57.56	51.19	49.80	49.97	51.41	6.9%	102.8%
	Medium	500	505.5	526.3	499.1	487.0	494.5	502.5	3.0%	100.5%
	High	5000	5201	5017	4768	5286	5297	5114	4.4%	102.3%
NOXC	LLOQ	50	51.28	55.52	54.80	52.18	56.15	53.99	4.0%	108.0%
	Low	100	105.71	103.41	97.49	106.40	101.76	103.0	3.5%	103.0%
	Medium	500	498.5	480.8	502.3	488.5	495.6	493.1	1.7%	98.63%
	High	5000	4860	4958	5008	5150	5085	5012	2.2%	100.2%
OXC	LLOQ	50	49.67	54.02	49.61	45.29	48.85	49.49	6.3%	98.98%
	Low	100	96.09	107.02	100.27	100.37	95.91	99.93	4.5%	99.93%
	Medium	500	484.2	498.0	518.4	479.2	489.8	493.9	3.1%	98.78%
	High	5000	4808	4945	4921	5155	5084	4983	2.8%	99.65%

of three measurements (i.e. peak areas resulting from LC–MS/MS) of an aqueous mixture of the analytes, using an elution time of 50 s. Matrix interferences were monitored by analyzing blank plasmas.

As depicted in Fig. 5, chromatograms of blank plasma samples were free of interferences from 15% methanol. Importantly, 15% methanol did not jeopardize its recovery or any of the three other compounds of interest (Fig. 6). Moreover, experiments showed that pH and buffer concentration are also crucial. Indeed, the same buffer at a 10 mM concentration, or ammonia at 0.1% and 0.5% showed a much faster decrease of NOXM recovery as the methanol content increased, and were thus not suitable (data not shown). As a result, clean-up was performed using ammonium buffer, 50 mM, pH 9.25, in H_2O/MeOH 85/15 (v/v).

3.3.4. Elution from trapping to analytical column

After sample clean-up, the trapping cartridge is set on-line with the analytical column for LC–MS/MS analysis of analyte elution. The duration of this coupling should be as short as possible to minimize the elution of plasma components onto the analytical column, which can impact the long-term performance of the assay. It was evaluated by monitoring elution of analytes from the trapping cartridge in the absence of the analytical column, while running the RP-LC gradient (Fig. 7). As described in the chromatography section, formic acid was used as pH-modifier. Besides its well-known role to promote ionization of solutes, it was also employed for analyte elution purposes. Indeed, considering $\log D_{calc}$ of analytes, changing the pH from alkaline to acidic will help with elution of analytes from the trapping cartridge. In this sense, the assay relies on a double gradient: in organic solvent and in pH. An elution time of 50 s was found to ensure a complete elution of all analytes, irrespective of the methanol content in the clean-up solution.

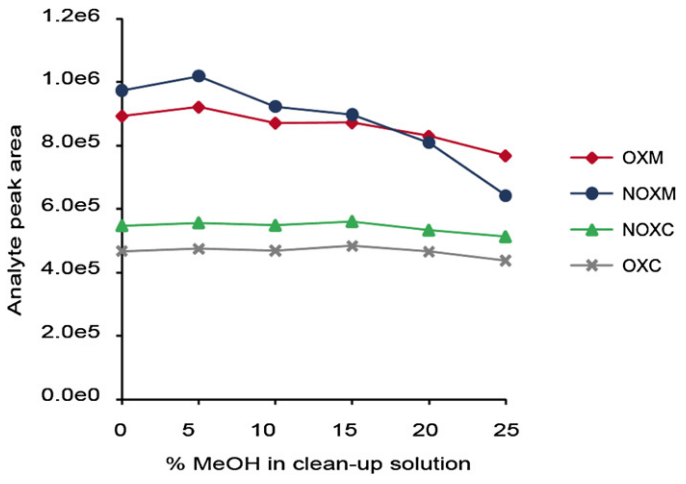

Fig. 6. Recovery of analytes as a function of the methanol content of the clean-up solution.

3.4. Validation

For validation purpose, five series of samples were analyzed on two different days. Each series was constituted by a set of 7 (NOXC and OXC) and 8 (NOXM and OXM) calibrator standard samples and four quality control (QC) samples. The corresponding levels of the latter were chosen as the lowest limit of quantification (LLOQ), 2 times LLOQ (QC low), 500 pg/ml (QC med) and 5000 pg/ml (QC high).

Fig. 8 shows representative chromatograms of blank plasma (P00) and spiked plasma at the lowest limit of quantification (LLOQ).

Using $1/x^2$ weighted least square regression, the assay was found to be linear from 25 to 10,000 pg/ml for NOXM and OXM, and

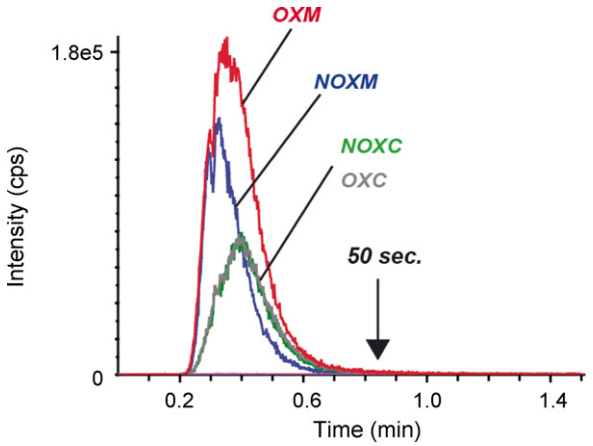

Fig. 7. Elution profile of the analytes in the absence of an analytical column.

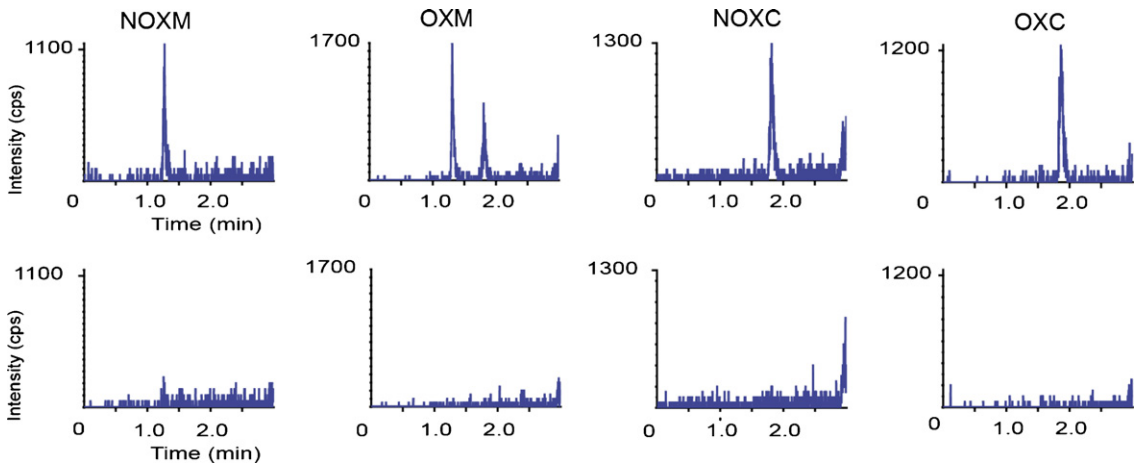

Fig. 8. Chromatograms of LLOQ level (upper trace) and P00 samples (lower trace).

Table 4
Accuracy for seven different plasmas spiked at 100 pg/ml.

	NOXM	OXM	NOXC	OXC
Plasma #1	97.0%	94.6%	96.5%	97.2%
Plasma #2	105%	103%	98.0%	94.7%
Plasma #3	110%	94.0%	94.0%	95.4%
Plasma #4	107%	96.6%	90.0%	98.8%
Plasma #5	90.9%	102%	87.8%	89.5%
Plasma #6	87.8%	101%	91.9%	98.4%
Plasma #7	101%	97.8%	98.9%	96.5%

from 50 to 10,000 pg/ml for NOXC and OXC. Precisions ($n = 5$) were better than 7% and accuracies were in the 98–108% range (Table 3).

As discussed above, the variability between samples in terms of endogenous interferences makes it mandatory to address the question of the selectivity of the assay. The latter was assessed by analyzing seven different human blank plasmas. No matrix interferences were observed on the SRM traces of analytes and IS (chromatograms not shown). Using column-switching methods, quantitative determination of analyte recovery and matrix effects can be challenging. Also when matrix effects occur between samples the internal standard should compensate for it. The only limitation is that a potential loss of signal should not affect precision and accuracy at the low calibration level. To investigate matrix effects seven different human blank plasmas were spiked at a concentration of 100 pg/ml, which represents a low level QC. Accuracies were found to be comprised between 87 and 110%, which suggests that no significant matrix effect impacted the analytical result (Table 4).

In conjunction with post-run cartridge washing, PPT allowed more than 200 plasma samples to be analyzed with a single trapping cartridge without compromising the performance of the assay. Moreover, if large batch analysis is required, the use of several cartridges does not compromise the quality of the data. Indeed, in addition to the samples of validation study (i.e. five series of calibrator standard and QC samples), two series of QC samples were analyzed using a second cartridge. Accuracies of the two latter QC series were calculated using the calibration curves obtained with the validation samples (i.e. using the first cartridge), and were found to be in the 92–106% range (data not shown). Finally, regarding the use of several cartridges, the Prospekt II system offers the advantage of fully controlling the column-switching process, as well as the possibility of using multiple cartridges if batches of several hundred samples need to be analyzed. In conclusion, the assay satisfied usual requirements for regulated bioanalysis.

3.5. Reanalysis of incurred samples

The validation of a bioanalytical method is based on the evaluation of several fundamental parameters, such as accuracy, precision, selectivity, sensitivity, reproducibility and stability [34]. Those are mostly estimated by the analysis of standard and QC samples, which should be prepared to be as representative as possible of the study (or incurred) samples intended to be measured. For this reason, standard and QC samples are usually obtained by spiking the analyte(s) in a pool or individual samples of the same biological matrix as the incurred samples (human plasma in the present work). Nevertheless, the composition of the resulting samples may not reflect those of the incurred samples. Inter-individual variations, especially in human samples, can indeed lead to differences in terms of matrix background (responsible for ionization and matrix effect issues) and/or matrix interferences (possibly metabolites). Under these conditions, the assessment of the performance of the method would be erroneous. It is nowadays accepted that the validation of a method should not be set rigidly solely in a pre-study framework, but should rather be a "living", on-going process, which could overlap the study phase. In fact there is a growing trend to include study samples for validation purposes.

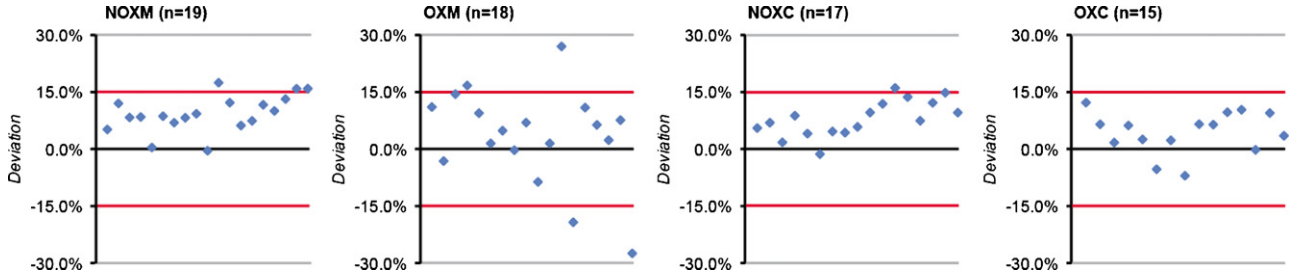

Fig. 9. Incurred sample reanalysis: deviation of the replicate from the original analysis.

Such approaches have been reported to reinforce confidence in the method [35,36]. The reanalysis of incurred samples, in particular, has been suggested to evaluate the reproducibility of the method in the current matrix and is the subject of increasing attention from the bioanalysis community [37,38].

In our evaluation, 20 samples were analyzed in duplicate, on two different days. For some samples and analytes, some results were found to be out of the limits of quantitation, and were, in consequence, not reported. This explains, from one analyte to another, different numbers (from 15 to 19) of measurements. Considering the 3rd AAPS/FDA Bioanalytical Workshop recommendations ("2 samples per subject for 10 subjects in a study") [37]), the latter were nonetheless sufficiently numerous for a rigorous evaluation.

For the interpretation of results, the first determination was considered as those of reference. The deviation from the second to the first measurement was then calculated and expressed as a percentage. The acceptance criterion was the classical "2-3-15 rule", which states that at least 2/3 of the replicate determinations should not deviate from the original value more than ±15%. Since these requirements were satisfied for all analytes (Fig. 9), the assay was in consequence found to be reproducible for the determination of study samples.

4. Conclusion

An automated, column-switching strategy was developed for the sensitive and simultaneous determination of NOXM, OXM, NOXC and OXC in human plasma. Sample preparation involved protein precipitation with perchloric acid, in order to allow multiple uses (>200) of a single SPE cartridge. Since the polarity of the analytes is pH-dependent, their retention on the cartridge was achieved using a polymeric, pH-resistant stationary phase and alkaline buffer for clean-up. Matrix interferences were removed by adding methanol to the previous clean-up solution. The elution of the analytes and the baseline separation of the isobaric species OXM and NOXC was then achieved using an acidic gradient. The selectivity of the assay was investigated with the analysis of seven different human plasmas, which were found to be free of interferences. The method was found to be linear from 25 to 10,000 pg/ml (OXM and NOXM) and from 50 to 10,000 pg/ml (OXC and NOXC), with precision and accuracy values meeting the usual requirements for regulated bioanalysis. Finally, the replicate analysis of incurred samples ensured the assay to be sufficiently reproducible to be employed for clinical, real-life studies.

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